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SerpinI2 (pancpin) is an inhibitory serpin targeting pancreatic elastase and chymotrypsin

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ABSTRACT

SerpinI2/Pancpin/MEPI is a 46 kDa member of the serpin (serine protease inhibitor) superfamily. It is downregulated in pancreatic and breast cancer, and associated with acinar cell apoptosis and pancreatic insufficiency when absent in mice. However, the target protease and protein properties of serpinI2 are previously uncharacterised. We have expressed and purified recombinant serpin I2 in *E. coli*. The protein exhibited thermal instability typical of inhibitory serpins, which was lost following RCL cleavage. SerpinI2 did not inhibit trypsin, but was found to inhibit pancreatic chymotrypsin and elastase with K_{ass} values $>10^5$ M^{-1} s^{-1}, and with stoichiometry of inhibition of 1.4 and 1.7 respectively. Mutagenesis of the predicted critical hinge region residue Ser344 abolished inhibitory activity, and a cleavage site C-terminal to Met358 was identified. The protein is also prone to polymerisation/aggregation at 45 °C, a characteristic of serpins associated with disease. This study therefore reveals a function for serpinI2 and supports the hypothesis that this protein can protect pancreatic cells from prematurely activated zymogens.

1. Introduction

The human protein pancpin (serpinI2) was originally identified as a gene downregulated in pancreatic cancer cells [1], encoding a 405 amino acid protein with significant sequence homology to serpin family members. This serpin was independently identified in human normal mammary gland as myoepithelial derived serine proteinase inhibitor or MEPI [2] and shown to have reduced expression in corresponding breast tumour tissue. No expression in malignant breast carcinoma was found, and transfection of MEPI into MD-MBA-435 cells inhibited *in vitro* invasion. Reduced plasminogen activation activity was reported in cells overexpressing pancpin/MEPI and suggested as a possible mechanism, but no direct inhibition of plasminogen activators was investigated [2]. The pancpin gene was found to cluster with the brain associated serpin gene, neuroserpin, at chromosome 3q26 with a shared gene organisation of 9 exons and 8 introns [3], and these proteins were subsequently assigned as the sole members of clade I in the human serpin nomenclature [4].

Loftus et al. [5] showed that serpinI2 deficiency in mice caused pancreatic insufficiency and acinar cell apoptosis, resulting in a stunted *pequeno* phenotype with immune cell defects and reduced life span. Reintroduction of the serpinI2 gene alone by bacterial artificial chromosome transgenic complementation could correct both acinar cell defects and immunodeficiency phenotypes. Dietary supplementation with pancreatic enzymes could also correct for growth retardation and immunodeficiency, despite continued acinar cell loss. Interestingly, the first identification of this serpin was a 405 amino acid homolog in rat pancreas (ZG-46p) that was shown to localise to zymogen granules and the Golgi complex of pancreatic acinar cells [6].

Despite a null mouse phenotype and a clear link to pancreatic function, the serpinI2 protein has not been purified from native or recombinant sources and no studies on inhibitory potential or target protease profile have previously been reported. Inhibitory serpins have a metastable native structure which allows a dramatic conformational change to a more stable state when either cleaved in the reactive centre loop (RCL) or when forming a complex with a target protease. This is facilitated by a flexible hinge region in the RCL, and the hinge region sequence of serpinI2, including a predicted P_{14} serine residue, suggests an ability to undergo the stressed to relaxed conformational change of inhibitory serpins. In this study we have expressed and purified recombinant serpin I2 in *E. coli*, which required removal of the predicted 18 amino acid hydrophobic signal sequence to obtain soluble 6xHis- tagged protein. The purified protein was examined for conformational thermal instability, screened for target protease specificity, and kinetic analysis of inhibition was performed.

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2. Experimental

2.1. Cloning of recombinant serpinI2

The full length cDNA encoding serpinI2 was obtained from RZPD (clone IRATp970C0944D). This was amplified and sub-cloned into the bacterial expression vector pRSET C (Invitrogen) using PCR primers designed to amplify a product coding for the mature protein (i.e. without the predicted signal sequence). Incorporation of BamH1 and EcoR1 sites allowed cloning into pRSETC in frame with the encoded N-terminal poly-His tag sequence. The expression vector insert was fully sequenced (GATC.de) to confirm human *SERPINI2* cDNA corresponding to the mRNA transcript NM_001012303.

2.2. Expression and protein purification

Heterologous protein expression from selected transformants in SoluBL21 cells (Gelantis) was optimised at 37 °C growth in autoinducing media (Formedium) containing ampicillin (0.1 mg/ml). SerpinI2 expression was detected using either anti-His tag antibody or anti-serpinI2 (R&D systems). Expressed protein was extracted from cell pellets using B-PER protein extraction reagent (ThermoScientific) and centrifuged at 20,000*g* for 30 min. The supernatant was applied to an immobilised nickel ion affinity chromatography column and purified as previously described for serpinB3 [7]. Eluted protein was concentrated by ultrafiltration using a 30 kDa MWCO membrane and prior to further analysis any aggregated or polymerised protein was removed by ultrafiltration through a 100 kDa MWCO membrane.

2.3. Thermostablity

Purified recombinant serpinI2 was partially cleaved by incubation with papain (10:1 M ratio) such that approximately equal amounts of intact and RCL clipped protein were obtained. The protein was incubated at increasing temperatures ranging from 30 °C to 90 °C for 100 min. Denatured protein was removed by centrifugation at 13,000*g* for 5 min and remaining soluble protein was analysed by SDS-PAGE. Circular Dichroism Spectroscopy was also used to examine serpinI2 conformational thermostability. Both intact serpinI2 (2 μM) and elastase cleaved serpinI2 were incubated at increasing temperatures (2 °C/min) in 20 mM sodium phosphate buffer pH 7. Structural stability was monitored at 222 nm using a Jasco J-810 Spectropolarimeter.

2.4. Protease inhibition assays and kinetic analysis

Initial screening for target proteases was performed using a 50-fold molar excess of serpin incubated at 37 °C for 10 min with a range of proteases. BSA (1 mg/ml) was included in the incubation to prevent autolysis of proteases, and residual activity compared to protease alone was determined using appropriate fluorogenic peptide substrates in each case. Kinetics of inhibition for bovine pancreatic α-chymotrypsin (E.C. 3.4.21.1) and porcine pancreatic elastase (E.C. 3.4.21.36) were performed using a discontinuous method to determine second order inhibition rate constants. Increasing concentrations of serpinI2 (100–400 nM) were incubated with 20 nM of either chymotrypsin or elastase. The decrease in residual activity with time was monitored using either Z-AAPF-AMC (30 μM) for chymotrypsin or Met-O-Succ-AAPV-AMC (30 μM) for elastase. The natural logarithm of residual activity against time was plotted to obtain the observed rate of inhibition (gradient $= -k_{obs}$). The association rate constant, k_{ass} ($M^{-1}\ s^{-1}$) was then determined from the slope of a plot of k_{obs} against inhibitor concentration.

To determine the stoichiometry of inhibition (SI) with chymotrypsin and elastase, recombinant serpinI2 was incubated with each protease at varying ratios for 30 min at 37 °C. Residual activity was again determined by monitoring cleavage of fluorogenic substrates, and the residual activity plotted against the ratio of inhibitor to enzyme ($[I]_0/[E]_0$). Linear regression analysis was used to determine the x-intercept which indicates the SI value.

2.5. SDS-stable complex formation

Following purification by IMAC chromatography, imidazole was removed from serpin I2 protein by desalting (Pierce G-25 desalt spin column) into PBS, 0.1% glycerol buffer. The protein was incubated at a 3:1 M ratio with either pancreatic chymotrypsin or elastase at 22 °C. Aliquots were taken at time intervals and immediately incubated at 95 °C with SDS-PAGE sample buffer. Complexes were visualised by Coomassie staining following 12% SDS-PAGE gel electrophoresis.

2.6. Mutagenesis of predicted P_{14} residue

Serine 344 was mutated to an Arginine residue using the Quikchange™ method (Stratagene) with the following primer pair (forward: 5′-atgaagatggtagagaagctgcaacatc-3′, reverse: 5′-gatgttgcagcttc tctaccatcttcat-3′). The plasmid insert was sequenced (GATC Biotech) to confirm incorporation of modified nucleotides.

2.7. Identification of cleavage site

Recombinant serpinI2 (2 μM) was incubated with either chymotrypsin or elastase (200 nM) at 37 °C for 5 min and the reaction stopped by the addition of PMSF (1 mM). Samples were subjected to SDS-PAGE, Coomassie stained, and the material deemed to be cleaved in the RCL region (approx 42.5 kDa) was excised. In-gel alkylation followed by tryptic digestion was performed essentially as described [8]. The samples were run on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. A high resolution (70,000) MS scan (350–1600 Da) was performed using the Q Exactive to select the 15 most intense ions prior to MS/MS analysis using Higher-energy C-trap dissociation. A stepped normalized collision energy was used to fragment selected ions. The raw data was *de novo* sequenced and searched against the Homosapien subset of the Uniprot Swissprot database using the search engine PEAKS Studio 6, for peptides cleaved with no specific enzyme.

2.8. Polymerisation potential

Polymerisation of wild-type serpinI2 was induced by incubation at 45 °C in 50 mM Tris-HCl, 150 mM NaCl at pH 6 or pH 8. Prior to incubation purified protein was filtered through a 100 kDa MWCO membrane to ensure predominantly monomeric serpin. Aliquots were taken at time intervals up to 60 min, and analysed by native PAGE.

3. Results

Initial attempts to express and purify protein from the full length *SERPINI2* gene encoding a 405 amino acid product were unsuccessful due to protein insolubility. Removal of the N-terminal 18 amino acid sequence, introduction of an N-terminal 6xHis fusion tag, and use of SoluBL21-DE3 (a derivative of BL21-DE3 cells generated by directed evolution to increase soluble protein expression) facilitated successful expression of soluble serpinI2. Expressed protein was purified from *E. coli* extracts by IMAC chromatography as previously described for B-clade serpins [7].

The purified protein was initially examined for an apparent change in stability following RCL cleavage, as is characteristic of inhibitory serpins able to undergo a stressed to relaxed conformation. Using a mixture of cleaved and intact serpin, we found that thermostability was lost at 50 °C for the intact serpin resulting in denatured precipitated protein that was removable by centrifugation when analysed by SDS-PAGE (Fig. 1a). Thermostability increased to 80 °C following RCL cleavage. Similarly, CD spectroscopy was performed on separate samples of intact or